Sphingolipids in bean leaves (*Phaseolus vulgaris*)

H. E. CARTER and J. L. KOOB

Division of Biochemistry, Department of Chemistry and Chemical Engineering, University of Illinois, Urbana, Illinois 61801

ABSTRACT Phytoglycolipid has been isolated for the first time from plant leaves (*Phaseolus vulgaris*). The purified product (almost identical with the phytoglycolipid isolated from flax seed) was a ceramide attached through phosphate diester linkage to an oligosaccharide, which consisted of the usual trisaccharide unit (inositol, hexuronic acid, hexosamine) to which were attached mannose, galactose, and arabinose. The major fatty acids were the saturated 2-hydroxy C_{22}, C_{24}, and C_{26} acids; the major long-chain bases were dehydrophytosphingosine (D-*ribo*-1,3,4-trihydroxy-2-amino-8-*trans*-octadecene) (53%) and phytosphingosine (D-*ribo*-1,3,4-trihydroxy-2-amino-octadecane) (32%).

A ceramide and a cerebroside were also isolated. In the ceramide the major fatty acids and the major long-chain bases were the same as in the phytoglycolipid. In the cerebroside, the fatty acid composition was similar to that in the ceramide and phytoglycolipid, but the long-chain bases consisted of dehydrophytosphingosine and phytosphingosine (7:1) with a substantial amount of unidentified long-chain base. The sugar component was glucose.

SUPPLEMENTARY KEY WORDS phytoglycolipid · cerebroside · ceramide · composition

EXTENSIVE studies have been made on the isolation and characterization of phytoglycolipid (PGL) from a wide variety of plant seeds (1–8). The presence of sphingolipid in the leaves of higher plants was established by Sastry and Kates (9), who isolated a glucose-containing cerebroside from the primary leaves of the scarlet runner bean (*Phaseolus multiflorus*). Allen et al. (10) isolated cerebroside from spinach leaves. Hirayama (11) detected cerebroside in both the total leaf lipids and chloroplast chlorophyll lipoprotein from *Sapium sebiferum* Roxb.

In 1962 Gladyshev (12) reported the isolation from bean and potato leaves of a lipopolysaccharide that he concluded was similar to the one isolated from Gram-negative bacteria. However, his data did not preclude that this material might be or might contain phytoglycolipid.

This paper reports the isolation and partial characterization of phytoglycolipid, ceramide, and cerebroside from the leaves of the bean (*Phaseolus vulgaris*).

METHODS

General

Unless otherwise specified, paper chromatograms were run descending on No. 1 Whatman filter paper in unlined tanks. Spots were detected by $AgNO_3$–NaOH dip, periodate–benzidine dip, acid aniline phthalate spray, or ninhydrin.

Phospholipids were chromatographed descending on formaldehyde-treated paper (13) in *n*-butanol–acetic acid–water 4:1:5 (upper layer) for 18 hr. Spots were detected with Rhodamine 6G under UV radiation, and were made permanently visible by counterstaining with Nile blue (14).

Plates coated with Silica Gel HR (Brinkmann Instruments, Inc., Westbury, N. Y.) were used for thin-layer chromatography. Spots were detected with 5% H_2SO_4 in methanol followed by charring, or with ninhydrin.

Gas–liquid chromatography was done on an F & M model 400 instrument equipped with a hydrogen flame ionization detector and a 2-m glass column, 4 mm I.D.,

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Abbreviations: PGL, phytoglycolipid; LCB-N, long-chain base nitrogen; BAW, *n*-butanol–acetic acid–water; CMW, chloroform–methanol–water; C-M, chloroform–methanol; DEAE, diethylaminoethyl.

packed with 3.8% SE-30 (methyl silicone) on Diatoport S, 80–100 mesh (F & M Scientific Corp., Avondale, Pa.). The fatty acid and long-chain base derivatives were tentatively identified by cochromatography with standards or from plots of log retention values (relative to an internal standard) against carbon number. The peak areas were estimated by multiplication of peak height by peak width at half-height.

The long-chain base nitrogen (LCB-N) was determined by the method of Lauter and Trams (15) with phytosphingosine as a standard. Total nitrogen was determined by the micro-Kjeldahl or Dumas method. Phosphorus was determined by the method of Bartlett (16). Sugars were determined by the orcinol method (17), with galactose as the standard. Hexosamine was quantified by the method of Elson and Morgan (18) as modified by Rondle and Morgan (19) and Boas (20), with glucosamine-HCl as the standard. Hexuronic acid was determined by the method of Dische as modified by Bitter and Ewins (21), with glucuronic acid as the standard.

Isolation

The green bush bean plants (*Phaseolus vulgaris* "Topcrop"), grown out of doors and without the use of insecticides, were uprooted when they reached the flower-bud stage (31–40 days after planting). The blades and leaflets were excised, washed, and stored in acetone at 10°C.

The leaves were drained and most of the adherent acetone was allowed to evaporate. The leaves were extracted as described by Gladyshev (12). The leaves (about 200-g dry weight) were blended with 2 liters of cold 0.1 N aqueous acetic acid in a chilled stainless steel Waring Blendor at medium speed for 2 min. The slurry was filtered through 18 layers of acid-washed cheesecloth on a large Büchner funnel under vacuum. The residue was extracted twice again in the same manner. Th equeous acetic acid filtrate contained no measurable LCB-N and so was discarded. The residue was air-dried overnight and was then refluxed with 2 liters of hot 70% ethanol (0.1 N in HCl) for 20 min. The slurry was filtered hot through cheesecloth washed with acidic ethanol. The residue was refluxed for 10 min twice more with 2-liter portions of acidic ethanol. The combined filtrates were chilled immediately and left at −10°C overnight. The precipitate was removed by centrifugation at 4080 *g* at 3–5°C for 20–30 min. The precipitates were washed with cold acidic ethanol, then with acetone until the washes were colorless, and finally with diethyl ether to yield a nonhygroscopic, tan powder. The typical yield of this crude extract was 1–3% of the dry weight of the leaves; analytical data are shown in Table 1. This material was insoluble in chloroform, methanol, and mixtures of the two; it was partially soluble in pyridine and 10% aqueous pyridine, and almost completely soluble in dimethyl sulfoxide and in the two phases of *n*-butanol–acetic acid–water (BAW) (4:1:5). Chromatography of this material in BAW 4:1:5 (upper phase) on formaldehyde-treated paper showed a spot with the same R_f as flax seed PGL (0.35–0.54).

TABLE 1 ANALYTICAL DATA FOR CRUDE AND PARTIALLY PURIFIED BEAN LEAF EXTRACT

	P	N	LCB-N	Sugar	Hexosamine
			%		
Crude extract	0.65	9.75	0.15	14.8	1.3
Crude sphingolipid fraction (Na^+)	0.84	4.43	0.42	10.8	2.4

When the extraction was done with a lower concentration of HCl (0.03%), the yields in both weight and LCB-N were much lower.

The crude extract (10.0 g) was dissolved in 100 ml each of upper and lower layer of BAW 4:1:5. A four-transfer countercurrent distribution was carried out. The layers were separated. The upper (less polar) layers U_1 and U_2 and lower layer L_4 were combined to yield 2.57 g of crude sphingolipid fraction.

Of this crude sphingolipid fraction 2.42 g was dissolved in 15 ml of pyridine to which 125 ml of water was added, and the solution was passed over a 2 × 61 cm column of the chelating ion-exchange resin Chelex 100, 50–100 mesh, Na^+ (BioRad Laboratories) (22). The material was quantitatively eluted with 1 liter of water; this yielded a crude sphingolipid fraction containing Na^+ as the major counterion. Analytical data for this material are shown in Table 1.

Purification of PGL on Diethylaminoethyl Cellulose

The high nitrogen content and strong amide absorption in the infrared spectrum of the partially purified extract suggested the presence of a considerable amount of peptides as impurities. Since silicic acid column chromatography did not separate this nitrogenous impurity from the PGL, we thought that diethylaminoethyl (DEAE) cellulose might offer more promise. The strong net negative charge due to the phosphate group of PGL along with some negative charge contribution from the hexuronic acid would cause PGL to be greatly retarded on this column while noncharged and less negatively charged molecules would be eluted first. Crude flax seed PGL, which had long-chain base and fatty acid compositions similar to those of crude bean leaf PGL, was therefore applied to a DEAE-cellulose column.

Flax Seed. A 50-g (3 × 60 cm) column of DEAE-cellulose (Selectacel reagent ion-exchange cellulose No, 70, 0.93 meq/g capacity, Schleicher and Schuell.

Keene, N. H.) was prepared as described by Rouser, Kritchevsky, Heller, and Lieber (23). 2 volumes of glacial acetic acid were passed over the column, followed by absolute methanol until the washes were neutral. The column was washed with chloroform and then with chloroform–methanol–water (CMW) 20:9:1. A 476-mg sample of crude flax seed PGL (Na^+) was suspended in 5 ml of chloroform and applied to the column. The linear gradient for elution consisted of 1 liter of CMW 20:9:1 in the mixing chamber and 1 liter of the same solvent, 0.6 M in ammonium acetate, in the second reservoir (24). 10-ml fractions were collected and P was determined in a 0.1-ml portion of every second fraction.

The contents of the tubes were combined into five major fractions (designated F 1–5) according to the phosphorus content. When these fractions were chromatographed on formaldehyde-treated paper, fraction F-4 (tubes 108–138) appeared to contain only PGL (R_f 0.30–0.50) and ammonium acetate. Fractions F-3 and F-5 contained little or no PGL; this indicatd a clean separation. The phosphatidyl inositol, a major contaminant in the crude flaxseed PGL, was eluted in fraction F-2 (tubes 77–86).

A 50-g 2.5 × 40 cm column of Sephadex G-25 (coarse) (Pharmacia Fine Chemicals, Inc., New Market, N. J.) was prepared for the desalting of fraction F-4 obtained from the DEAE-cellulose column, as described by Siakotos and Rouser (25). This PGL fraction (160 mg) was suspended in 5 ml of chloroform–methanol 19:1 saturated with water (Solvent 1) and applied to the Sephadex column, which was eluted with 500 ml of the same solvent. The column was successively eluted with 1 liter of the lower phase of a mixture of five parts of chloroform–methanol 19:1 (850 ml) and one part of glacial acetic acid (170 ml) to which was added 25 ml of water (Solvent 2); 500 ml of the lower phase of a mixture of five parts of chloroform–methanol 9:1 (425 ml), and one part of glacial acetic acid (85 ml) to which was added 21 ml of water (Solvent 3); and one liter of methanol–water 1:1 (Solvent 4) (25). In this procedure Solvent 1 elutes most lipids but very little PGL; Solvent 2 elutes the PGL. Solvents 3 and 4 elute water-soluble nonlipid substances (such as ammonium acetate) that are discarded. After the column had been allowed to stand in Solvent 4 for 48 hr it was washed with 500 ml of Solvent 1 and was ready for re-use. When F-4 was desalted on Sephadex, elution with Solvent 1 yielded a small amount of impure material, which was discarded; elution with Solvent 2 yielded 105 mg of PGL. The latter was desalted a second time in the same manner to remove the remaining ammonium acetate. Analytical data for this purified flax seed PGL are shown in Table 2.

Bean Leaf. A 180-g DEAE-cellulose column (5 × 60 cm) was prepared as before. The bean leaf crude sphingolipid (Na^+) (2.35 g) was suspended in 20 ml of chloroform–methanol–water 20:9:1 and applied to the column, which was eluted as described above but with 4 liters of each solvent. 20-ml fractions were collected and phosphorus was determined on 0.1 ml of every second tube. The contents of the tubes were combined into eight major fractions (designated B 1–8) according to the phosphorus content, and desalted on Sephadex G-25. When necessary, the fractions were passed over a Sephadex column a second time to remove all the ammonium acetate. Portions of each major fraction were chromatographed on formaldehyde-treated paper. Fraction B-5 (141 mg, desalted) (tubes 173–217 from the DEAE-cellulose column) contained most of the PGL (R_f 0.2–0.4). Fraction B-4 contained very little PGL but fractions B-6 (tubes 218–220), B-7 (tubes 221–290), and B-8 (tubes 291–337) contained a considerable amount of PGL as evidenced by paper chromatographic behavior and analyses. Analytical data for the major fractions are shown in Table 3. The structural studies were done on desalted fraction B-5.

The purified flax seed and bean leaf PGL (desalted fractions F-4 and B-5, respectively) were fluffy, white, nonhygroscopic powders that formed opalescent solutions in hot water and were soluble in pyridine and 10% aqueous pyridine. Each gave a single spot when chromatographed on formaldehyde-treated paper. The specific rotation of the purified flax seed PGL was $[\alpha]_D^{28} = +56°$ (0.47 g/100 ml in pyridine); for the purified bean leaf PGL the specific rotation was $[\alpha]_D^{29} = +58°$ (0.49 g/100 ml in pyridine).

Structural Studies on Bean Leaf PGL

Throughout the following structural studies, equal

TABLE 2 ANALYTICAL DATA FOR FLAX SEED PGL (DESALTED ON SEPHADEX G-25) FROM FRACTIONATION OF DEAE-CELLULOSE

	P	N Total	N as NH_4^+*	LCB-N	Sugar	Hexuronic Acid	Hexosamine
				%			
Flax seed PGL after first desalting on Sephadex G-25	2.24	2.36	0.40	0.79	8.1	15.4	9.6
Flax seed PGL after second desalting on Sephadex G-25	2.14	—	—	0.90	—	—	—
Theory for PGL (Tetrasaccharide)	2.18	1.96	—	0.98	—	13.6	12.6

* Determined by the micro-Kjeldahl procedure, omitting the acid digestion.

TABLE 3 ANALYTICAL DATA FOR SOME OF THE DESALTED FRACTIONS FROM FRACTIONATION OF BEAN LEAF CRUDE SPHINGOLIPID (Na^+) BY DEAE-CELLULOSE COLUMN CHROMATOGRAPHY

Fraction No.	Weight	P	N	LCB-N	Sugar	Hexuronic Acid	Hexosamine
	mg	%	%	%	%	%	%
B-1	259	0.03	1.33	1.18	1.98	—	—
B-5	141	1.86 1.84*	3.32 2.47*	0.88 0.94*	13.9	14.0	9.9
B-6	13	1.68	—	0.78	—	—	—
B-7	111	1.42	—	0.64	17.7	15.3	4.7
B-8	42	1.25	—	0.54	—	12.3	2.9

* Value obtained after fraction B-5 was desalted a second time on Sephadex G-25.

amounts of flax seed PGL were carried through the same procedures to act as controls.

Bean leaf PGL (5.3 mg) was hydrolyzed with 0.5 ml of 0.5 N sulfuric acid in a sealed tube at 100°C for 12 hr. The hydrolysate was centrifuged and the precipitate washed with water. The supernatant fractions were neutralized with Dowex 2 (HCO_3^-), a weak anion-exchange resin, and chromatographed on paper in ethyl acetate–acetic acid–water 3:1:3, upper phase. The paper chromatogram showed the presence of galactose, mannose, and arabinose. The precipitate was lyophilized and then methanolyzed in 2.5 ml of 1 N methanolic HCl (prepared by adding 8.6 ml of concentrated HCl to 12.6 ml of water and adding absolute methanol to make 100 ml) for 16 hr at 75–80°C (26). The fatty acids and long-chain bases, respectively, were isolated from the hydrolysate by the method of Kates (27).

The fatty acids were esterified with diazomethane and analyzed by gas–liquid chromatography at 240°C. The identification of the fatty acids was further confirmed by gas–liquid chromatography of the trimethylsilyl ethers of the hydroxy fatty acid methyl esters. These derivatives were prepared by the addition of 0.1 ml of silylation reagent (28) to a hexane solution of a 0.5 mg portion of the methyl esters. After one hr the trimethylsilyl derivatives were chromatographed at 240°C. The area percentages were in good agreement in the two methods used for analysis of the fatty acids. The fatty acid composition is shown in Table 4.

The long-chain base fraction, chromatographed by thin-layer chromatography (28) in chloroform–methanol–2 N NH_4OH 40:10:1 gave ninhydrin-positive spots corresponding to phytosphingosine and (or) dehydrophytosphingosine and two spots in the region of the 1,4-anhydro derivatives of these bases. The trimethylsilyl derivatives of the long-chain bases were prepared by addition of enough silylation reagent (29) to make a 0.3–0.6% (w/v) solution. After 10–15 min the trimethylsilyl derivatives of the long-chain bases were analyzed by gas–liquid chromatography at 209°C. The long-chain base composition is shown in Table 5.

TABLE 4 FATTY ACIDS OF BEAN LEAF AND FLAX SEED PHYTOGLYCOLIPIDS AND BEAN LEAF CERAMIDE AND CEREBROSIDE

Fatty Acid	Phytoglycolipid		Ceramide	Cerebroside
	Bean Leaf	Flax Seed	Bean Leaf	Bean Leaf
		area %		
16h:0*	—	4	—	1
20:0	—	—	—	3
22:0	1	—	—	4
22h:0	20	7	13	15
24:0	4	2	—	—
23h:0	6	7	6	4
24h:0	48	44	59	42
25h:0	6	17	7	4
26h:0	10	11	15	12
Unidentified or minor	6	8	—	14

* 16h:0 = 2-hydroxyhexadecanoic acid.

Bean leaf PGL (52.6 mg) was hydrolyzed with 10 ml of saturated aqueous barium hydroxide under reflux for 8 hr (2). The hydrolysate was left at room temperature overnight and was subsequently filtered. The filtrate and washes were passed over a 15-ml column of the weak cation exchanger Amberlite IRC-50 (H^+) and the column was eluted with 4–5 volumes of water. The eluate was brought to pH 4–5 with Dowex 2 (HCO_3^-) and then passed over a small column of fresh Dowex 2 (HCO_3^-), the used resin being added to the top of the column (1.2 × 15 cm, total). The phosphorus-free oligosaccharides were eluted with 200 ml of water and lyophilized to yield 8.2 mg. The phosphorylated oligosaccharides were eluted from the Dowex 2 column with 5% ammonium acetate, lyophilized, and desalted on Sephadex G-10 to yield 3.3 mg of white powder. Flax seed PGL (Na^+) (54.8 mg), when treated as described above, yielded 11 mg of phosphorus-free oligosaccharide. No attempt was made to recover the phosphorylated oligosaccharide from the flax prepara-

TABLE 5 LONG-CHAIN BASE COMPOSITION OF FLAX SEED PGL AND BEAN LEAF PGL, CERAMIDE, AND CEREBROSIDE

Long-Chain Base	Phytoglycolipid		Ceramide	Cerebroside
	Flax Seed	Bean Leaf	Bean Leaf	Bean Leaf
			area %	
Anhydrodehydrophytosphingosine*†	7	7	14	2
Anhydrophytosphingosine†‡	3	6	6	2
C_{16}-phytosphingosine§	3	—	—	—
Dihydrosphingosine	—	2	—	4
Dehydrophytosphingosine	70	53	54	52
Phytosphingosine	17	32	24	7
Unidentified	—	—	—	32

* 2-(*trans*-tetradec-5-enyl)-3-hydroxy-4-amino tetrahydrofuran.

† These anhydro-bases are thought to be degradation products resulting from acid methanolysis of phytosphingosine- and dehydrophytosphingosine-containing compounds.

‡ 2-tetradecyl-3-hydroxy-4-aminotetrahydrofuran.

§ D-*ribo*-1,3,4-trihydroxy-2-aminohexadecane.

tion. Paper chromatography of the bean leaf and flax nonphosphorylated oligosaccharide in *n*-butanol–pyridine–water 6:4:5 showed ninhydrin-positive, alkaline $AgNO_3$-positive spots corresponding to the trisaccharide glucosamido-glucuronido-inositol and a slower-moving spot corresponding to a slightly longer oligosaccharide as well as a faint ninhydrin-positive spot in the region of inositol and (or) disaccharide.

Bean leaf nonphosphorylated oligosaccharide (6.7 mg) was hydrolyzed with 0.5 ml of 2 N sulfuric acid in a sealed tube for 30 min at 100°C. The hydrolysate was brought to pH 4–5 with Dowex 2 (HCO_3^-) and lyophilized. Paper chromatograms of the hydrolyzed oligosaccharide showed a spot corresponding to trisaccharide; spots corresponding to galactose, mannose, and (or) arabinose appeared, and the spot corresponding to the higher oligosaccharide had disappeared.

Charcoal (Darco G-60, Atlas Powder Co., Wilmington, Del.) (100 g) was stirred with a solution of 3 g of oleic acid in 400 ml of absolute methanol to which 4 liters of water had been added. The liquid was decanted and the charcoal was treated with fatty acid twice more. The charcoal was filtered and dried at 50°C. This treatment increased the recovery of sugars applied to charcoal columns. A 4 × 118 mm column of treated charcoal was packed as an aqueous slurry and washed with water. Hydrolyzed bean leaf nonphosphorylated oligosaccharide (5 mg), dissolved in 0.1 ml of water, was applied to the column. In a modification of the elution scheme of Whistler and Durso (30), the monosaccharides (2.5 mg) were eluted with 10 ml of water, and the trisaccharide (2 mg) with 3% ethanol. Elution with 15% ethanol yielded essentially nothing. The water eluate was chromatographed on paper in ethyl acetate–acetic acid–water 3:1:3, upper phase, and showed galactose, mannose, arabinose, and a trace of inositol (probably released in the barium hydroside hydrolysis). The trimethylsilyl derivatives were prepared from another portion of the water eluate and gas–liquid chromatography of these derivatives showed that the ratio galactose/arabinose/mannose was 1:2:1. The 3% ethanol eluate showed a single ninhydrin-positive spot, which corresponded to glucosamido-glucuronido-inositol ($R_{galactose}$ 0.22) when chromatographed on paper in *n*-butanol–pyridine–water 6:4:5. The hexuronic acid content of this material was 34%. This bean leaf trisaccharide (1 mg) was hydrolyzed with 0.1 ml of 6 N HCl for 6 hr in a sealed tube at 100°C. The hydrolysate was neutralized with Dowex 2 (HCO_3^-). Chromatography of this material in *n*-butanol–pyridine–water 6:4:5 showed two major spots, corresponding to glucosamine (ninhydrin-positive) and inositol, and some minor spots which were probably degradation products. A sample of glucosamido-glucuronido-inositol treated in the same manner showed the same pattern of spots.

In the assay procedure for hexosamine, the acid hydrolysate of a portion of the bean leaf PGL was passed over a Dowex 50 (H^+) (strong cation-exchanger) column. The water eluate was neutralized with Dowex 2 (HCO_3^-) and lyophilized. The trimethylsilyl derivative was prepared and gas–liquid chromatography at 180°C gave a single peak corresponding to *myo*-inositol.

Purification of Bean Leaf Ceramide and Cerebroside

The first bean leaf fraction, B-1, eluted from the DEAE-cellulose column, after desalting, had high LCB-N and ran much faster than PGL on formaldehyde-treated paper, which suggested a less polar sphingolipid. A portion of this material (78 mg) was dissolved in 1 ml of chloroform–methanol (C–M) 99:1 and was applied to a 30-g silicic acid column (Unisil, Clarkson Chemical Co., Williamsport, Pa.), 1.5 × 38 cm. The column was eluted with 400 ml each of C–M 99:1, C–M 98:2, C–M 95:5, C–M 80:20, and methanol. The fractions were monitored by thin-layer chromatography. A ceramide (17 mg, LCB-N 2.09%), eluted with C–M 95:5, showed a single spot by thin-layer chromatography in C–M 93:7 at R_f 0.43. Yeast ceramide gave an R_f of 0.38 in this system. The infrared spectrum was almost identical with that for yeast ceramide, except for the presence of strong absorption (970 cm^{-1}) by the *trans* double bond (31, 32) in the bean leaf ceramide.

The cerebroside [15 mg, LCB-N 1.46%, sugar 17% (glucose standard)] eluted from the silicic acid column with C–M 80:20, gave a major spot at R_f 0.28 and a minor spot at R_f 0.14 when subjected to thin-layer chromatography in C–M 9:1. Bovine brain "phrenosin" had R_f 0.30 in this system. This material gave an infrared

spectrum almost identical to that for the bovine brain "phrenosin," except for a weak absorption due to the ester-containing contaminant that corresponded to the minor spot at R_f 0.14 on the thin-layer chromatogram. The amount of this contaminant was decreased by subsequent silicic acid chromatography and alkaline hydrolysis, but it was not totally eliminated.

Structural Studies on Bean Leaf Ceramide and Cerebroside

A portion of the purified bean leaf ceramide (4 mg) was methanolyzed as described previously. The fatty acid and long-chain base compositions as characterized by gas–liquid chromatography are shown in Tables 4 and 5, respectively. Thin-layer chromatography of the long-chain bases in chloroform–methanol–2 N NH_4OH 40:10:1 showed ninhydrin-positive spots that corresponded to dehydrophytosphingosine and (or) phytosphingosine and the anhydro derivatives of these long-chain bases.

A portion of the slightly ester-contaminated cerebroside (2.5 mg) was methanolyzed and the resultant fatty acids, long-chain bases, and methyl glycosides were characterized by gas–liquid chromatography. The fatty acid and long-chain base compositions are shown in Tables 4 and 5, respectively. The methyl glycoside fraction, chromatographed as trimethylsilyl derivatives at 170°C, gave only two peaks; these cochromatographed with methyl α-glucoside and methyl β-glucoside, respectively.

DISCUSSION

Previous studies of leaf lipids obtained by the conventional chloroform–methanol extractions have resulted in the isolation of cerebrosides but not of ceramides or PGL. Whether these compounds were not extracted under the conditions used or whether they were not detected is not known. The acidic ethanol extraction procedure, used by Gladyshev (12) for the extraction of lipopolysaccharide from plant leaves, does extract all three of these sphingolipids. Although this procedure may not be optimal for extraction, the PGL isolated was similar in properties and molecular size to the PGL isolated from the crude phosphatides of various seeds. The ceramide could be an artifact produced from a more complex sphingolipid during the acidic extraction.

Column chromatography on DEAE-cellulose separated the other sphingolipids, the peptide contaminant, and the phosphatides from the PGL. The Sephadex column used to desalt the fractions from the DEAE-cellulose columns had an added advantage: trace amounts of phosphatides that were eluted from the DEAE-cellulose column with the first fraction of the PGL were eluted from the Sephadex column with Solvent 1 in the desalting procedure, where as the PGL was eluted mainly with Solvent 2.

The relatively broad spread of the bean leaf PGL on the DEAE-cellulose column could be due to several causes, among them the low solubility of the crude material in the chloroform–methanol–water solvent used to apply the sample to and elute the column. Another cause could be variations in the molecular size and oligosaccharide composition of the later fractions eluted from the DEAE-cellulose column. Analytical data for fractions B-7 and B-8 and the R_f values, slightly lower than that of the major PGL (B-5) on formaldehyde-treated paper, suggest a slightly longer oligosaccharide chain in B-7 and B-8. The greater retardation of this material on the DEAE-cellulose column, in spite of possibly greater molecular size, can be explained by the greater net negative charge due to the lower hexosamine content and relatively higher hexuronic acid content in fractions B-7 and B-8 than in B-5 and B-6.

The PGL of the crude extract constitutes 0.1% of the dry weight of the leaves (3–4% of the crude extract) and contains about 13% of the hexosamine in the leaves (29% of the hexosamine in the crude extract). The PGL contains about 26% of the LCB-N in the crude extract, which contained twice as much LCB-N as was in the leaves (as measured by the Lauter and Trams procedure, which seems to give only a rough estimate on crude materials). The bean leaf PGL fraction (B-5) used for structural studies amounted to approximately half the total PGL eluted from the DEAE-cellulose column.

The ceramide and cerebroside constituted at least 0.6% each of the crude extract and contained about 9 and 5% of the LCB-N, respectively, from the crude extract.

The fatty acid compositions of the three bean leaf sphingolipids are closely similar to one another and to that of the flax seed PGL. Although the long-chain base compositions of the bean leaf PGL and bean leaf ceramide were almost identical, the base composition of the cerebroside, in contrast, showed much less phytosphingosine relative to dehydrophytosphingosine and much unidentified long-chain base. Sastry and Kates (9) found an even higher dehydrophytosphingosine/phytosphingosine ratio (20:1) and reported 20% unidentified long-chain base in the cerebroside from runner bean leaves.

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References

1. Carter, H. E., W. D. Celmer, W. E. M. Lands, K. L. Mueller, and H. H. Tomizawa. 1954. *J. Biol. Chem.* **206:** 613.
2. Carter, H. E., W. D. Celmer, D. S. Galanos, R. H. Gigg, W. E. M. Lands, J. H. Law, K. L. Mueller, T. Nakayama, H. H. Tomizawa, and E. Weber. 1958. *J. Amer. Oil Chem. Soc.* **35:** 335.
3. Carter, H. E., R. H. Gigg, J. H. Law, T. Nakayama, and E. Weber. 1958. *J. Biol. Chem.* **233:** 1309.
4. Carter, H. E., D. S. Galanos, H. S. Hendrickson, B. Jann, T. Nakayama, Y. Nakazawa, and B. Nichols. 1962. *J. Amer. Oil Chem. Soc.* **39:** 107.
5. Carter, H. E., and H. S. Hendrickson. 1963. *Biochemistry.* **2:** 389.
6. Carter, H. E., S. Brooks, R. H. Gigg, D. R. Strobach, and T. Suami. 1964. *J. Biol. Chem.* **239:** 743.
7. Carter, H. E., B. E. Betts, and D. R. Strobach. 1964. *Biochemistry.* **3:** 1103.
8. Carter, H. E., D. R. Strobach, and J. N. Hawthorne. 1969. *Biochemistry.* **8:** 383.
9. Sastry, P. S., and M. Kates. 1964. *Biochemistry.* **3:** 1271.
10. Allen, C. F., P. Good, H. F. Davis, P. Chisum, and S. D. Fowler. 1966. *J. Amer. Oil Chem. Soc.* **43:** 223.
11. Hirayama, O. 1965. *J. Biochem. (Tokyo).* **57:** 581.
12. Gladyshev, B. N. 1963. *Biokhimiya.* **28:** 210.
13. Hörhammer, L., H. Wagner, and G. Richter. 1959. *Biochem. Z.* **331:** 155.
14. Rouser, G., A. J. Bauman, N. Nicolaides, and D. Heller. 1961. *J. Amer. Oil Chem. Soc.* **38:** 565.
15. Lauter, C. J., and E. G. Trams. 1962. *J. Lipid Res.* **3:** 136.
16. Bartlett, G. R. 1959. *J. Biol. Chem.* **234:** 466.
17. Svennerholm, L. 1956. *J. Neurochem.* **1:** 42.
18. Elson, L. A., and W. T. J. Morgan. 1933. *Biochem. J.* **27:** 1824.
19. Rondle, C. J. M., and W. T. J. Morgan. 1955. *Biochem. J.* **61:** 586.
20. Boas, N. F. 1953. *J. Biol. Chem.* **204:** 553.
21. Bitter, T., and R. Ewins. 1961. *Biochem. J.* **81:** 43P.
22. Carter, H. E., and E. J. Weber. 1966. *Lipids.* **1:** 16.
23. Rouser, G., G. Kritchevsky, D. Heller, and E. Lieber. 1963. *J. Amer. Oil Chem. Soc.* **40:** 425.
24. Hendrickson, H. S., and C. E. Ballou. 1964. *J. Biol. Chem.* **239:** 1369.
25. Siakotos, A. N., and G. Rouser. 1965. *J. Amer. Oil Chem. Soc.* **42:** 913.
26. Gaver, R. C., and C. C. Sweeley. 1965. *J. Amer. Oil Chem. Soc.* **42:** 294.
27. Kates, M. 1964. *J. Lipid Res.* **5:** 132.
28. Sambasivarao, K., and R. H. McCluer. 1963. *J. Lipid Res.* **4:** 106.
29. Carter, H. E., and R. C. Gaver. 1967. *J. Lipid Res.* **8:** 391.
30. Whistler, R. L., and D. F. Durso. 1950. *J. Amer. Chem. Soc.* **72:** 677.
31. Mislow, K. 1953. *J. Amer. Chem. Soc.* **74:** 5155.
32. Marinetti, G., and E. Stotz. 1954. *J. Amer. Chem. Soc.* **76:** 1345.
33. Wells, W. W., C. C. Sweeley, and R. Bentley. 1964. *In* Biomedical Applications of Gas Chromatography. H. A. Szymanski, editor. Plenum Press, New York.